Observations on an Intra-nucleolar Body in Columnar Epithelium Cells of the Intestine.

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With Plate 17 and 1 Text-figure.

Contents.

1. Introductory.

In the course of observations on the columnar epithelium cells of the cat's intestine, it was noticed that the Cajal formol-silver nitrate method for the Golgi apparatus sometimes demonstrated one or more intra-nucleolar granules. In this paper an attempt has been made to describe these bodies in the resting cell and their behaviour during mitosis.

The term "nucleolus" is somewhat indiscriminately applied to one or more bodies, usually rounded or oval, lying within the nucleus. When these structures are basophil in staining reaction they are termed "karyosomes" or "chromatin nucleoli"; when oxyphil, "plasmosomes" or sometimes "true nucleoli." Throughout this paper the term "nucleolus" is used as a general term comprising both karyosome and plasmosome, there being, so far as I am aware, no other word available—unless a new term were created, which does not seem desirable.

I am indebted to Mr. E. S. Goodrich for advice and criticism. I am also glad to take this opportunity for thanking him for his many kindnesses to me when a student. To Prof. C. S. Sherrington my warmest thanks are due for his interest in my work, while to my friend, Mr. J. Bronté Gatenby, formerly of this Department, I am indebted for training in cytological technique. Finally, part of the work was done in the Zoology Laboratory of University College, London, for which I have to thank Prof. J. P. Hill.

2. Previous Work.

In the abundant literature upon the nucleoli of animal cells, many references may be found to intra-nucleolar inclusions, especially in the nucleoli of oöcytes.

Some authors have observed intra-nucleolar granules in fixed and stained material (**3, 8**), while others have described vacuoles (**6, 11**). Finally, a few observations have been made upon the nucleoli of cells examined intra-vitam (**1, 2**). Montgomery, in a remarkable paper (**11**) upon the nucleolus, gives a chronological summary of the literature containing references to intra-nucleolar bodies and vacuoles. Unfortunately most of the observations on these have been made either upon fixed and stained tissues, or on cells examined intra-vitam. Many of the observations were made on material fixed and stained by the crudest methods. It is therefore difficult to distinguish between what may have been artefact and what was not.

The following references will illustrate the scope and nature of the observations hitherto made upon nucleoli:

Haeckel (17) states: "Endlich findet man in vielen Eiern (aber nicht in allen) innerhalb dieses Keimfleckes noch ein innerstes Pünktchen, einen Nucleolinus, welchen man Keimpunkt (Punctum germinativum) nennen kann."

Montgomery (11), summarising his personal observations, says: "In agreement with the majority of observers, I can attach no particular morphological significance to the nucleolinus; it appears to be only a detached portion of the nucleolar ground substance, to be in most cases absent, and when present to vary greatly in regard to size, position and number. It is undoubtedly the case that many structures which have been described as nucleolini are in reality minute vacuoles, which from their refrangibility appear to be granules. . . ."

Maziarski (10), in an interesting study of the nucleus of the intestinal epithelial cells in Isopod Crustacea, notes that plasmosomes frequently contain basophil granules which he homologises with the chromatin.

Prenant, Bouin and Maillard (12) say of the nucleolus: "Au lieu d'être homogène, comme c'est le cas habituel, il offre parfois une structure. . . . Bien d'autres complications structurales ont été signalées dans certains nucléoles; une membrane d'enveloppe, etc."

No observations, so far as I am aware, have been made upon nucleoli in material treated by the Cajal method for the Golgi apparatus, with the exception of those of P. del Rio Hortega (9). This worker has noted that in mammalian ova "la tâche germinative ou nucléole ovulaire apparait par les méthodes de l'urane comme constituée par des granulations argentophiles disposées périphériquement au nombre de quatre à six ou par petits bâtonnets alignés dans la direction transversale; le nucléole forme une espèce de petite vessie claire d'où se détachent vigoureusement ces granules." In some of the figures in Cajal's memoir (4) on the Golgi apparatus intra-nucleolar bodies are depicted, but no reference is made

to them.[1] Further, I am not acquainted with any observations on intra-nucleolar inclusions in intestinal epithelial cells of vertebrates either by the Cajal or any other method.

Throughout this paper the intra-nucleolar body studied has been termed a nucleolinus. There are at least eleven terms which have been applied to intra-nucleolar inclusions. Out of these I have chosen the word "nucleolinus," such a course seeming wiser than to burden cytological literature with yet another new term.

3. Material and Methods.

It was found that the formol-silver nitrate method of Cajal (4) sometimes demonstrated the nucleolinus of the columnar epithelial cells of the cat's small intestine with extraordinary clearness. In such preparations the nucleolinus was the only impregnated structure within the nucleus.

As counterstains hæmatoxylin and eosin (used according to Scott's method (13)), Mann's methyl blue-eosin, pyronin-methyl green, and tolnidin blue-eosin were used. For the study of the behaviour of the nucleolinus during mitosis, safranin gave the most valuable results, as its clearness of definition was found to be less affected by the silvering process than was the case with other stains.

In the cat the best preparations were obtained by using the Cajal method unmodified, the technique of which I have described elsewhere (5).

In the case of the frog, however, it was not found possible to demonstrate the nucleolinus by the standard Cajal method. After many attempts with various modifications of the original

[1] Since the above was written, Cajal's "Manual de Histología Normal" has come into my hands. In this very remarkable text-book, which those who regard mitochondria and Golgi apparatus as artefacts would do well to study, Cajal refers to nucleolini as being frequently artefacts. He adds, however, that within the nucleoli of nerve cells "se observan claramente espérulas interiores y una materia intersticial." [p. 201.]

the following technique gave almost invariably successful results:

(1) Fixation for ten to eleven hours in the uranium nitrate-formol mixture made up thus:

Uranium nitrate . . .	2 grm.
Formol (40 per cent.) . .	6 c.c.
Aq. dest.	100 ,,

(2) Rinsing the pieces of tissue in aq. dest. for a few minutes.

(3) Impregnation with a 3 per cent. solution of silver nitrate for thirty-six hours.

(4) Rinsing in aq. dest.

(5) Reduction in the standard solution for one to one and a-half hours.

The material was then washed in water, dehydrated in ascending grades of alcohol, cleared with xylol, and imbedded in wax in the usual manner.

As may be seen by comparison with the ordinary Cajal method (**4**, **5**), the above technique differs from it in (A) the greater uranium nitrate content, 2 grm. instead of 1, and (B) the strength of the silver nitrate-solution—3 per cent. instead of 1·5 per cent. For reasons which I am unable to explain, the action of this modified technique for the nucleolini of the columnar cells of the intestine of the frog is such that these elements are generally the only impregnated structures within the cells, the Golgi apparatus being only very rarely demonstrated.

In addition to the original and modified Cajal methods, control observations on the nucleolini were made by ordinary cytological technique. Teased intestinal mucosa was also examined intra-vitam and with intra-vitam stains.

4. The Nucleolinus in the Resting-Cell.

(A) In the Cat.—In a successful Cajal preparation of the intestinal epithelium the following structures may be seen:

(i) The Golgi apparatus, appearing as a coarse, irregular

reticulum, impregnated black, the interspaces of the network clearer than the surrounding ground cytoplasm.

(ii) The mitochondria, impregnated golden to dark-brown. It is a curious fact that only the mitochondria in the outer portion of the cells (i. e. between the nucleus and the striated border) are demonstrated, the mitochondrial group lying between the nucleus and the base of the cells being never shown by this method, although Champy-iron hæmatoxylin preparations demonstrate its presence. That this curious selectivity is not due to the silver impregnation failing to penetrate the whole depth of the cells, is shown by the fact that this differentiation holds good for all the columnar cells throughout the thickness of the intestinal mucosa. It can probably be ascribed to chemical differences in the two mitochondrial groups.

(iii) The nucleolini, impregnated black, and sharply differentiated from the surrounding nucleolar substance.

Reference to Pl. 17, fig. 1, will make clear the various impregnated elements within the cell.

Cajal preparations counterstained with safranin show the nucleolini black, the nucleoli and chromatin pink. The nucleoli number from one to three—most generally two per nucleus. Each nucleolus may contain as many as five nucleolini, but the vast majority have but one or two. The intra-nucleolar bodies are usually round, more rarely stumpy rodlets, measuring approximately 0·2 to 0·5 μ in diameter. Reference to the nuclei drawn in Pl. 17, figs. 2 to 5, will illustrate this.

Now sections of Cajal material stained with pyronin-methyl green show the greater number of the nucleoli to be karyosomes, i. e. they stain with the methyl-green component like chromatin. A few of the nucleoli, however, stain more red than green, thereby approximating the staining reaction of plasmosomes. Observations on the same material counterstained with hæmatoxylin and eosin, used according to Scott's method (**13**), and Mann's methyl blue-eosin, give similar pictures of a gradation from basophility to oxyphility

in some of the nucleoli. Variations such as these have been noted within a single crypt.

Occasionally a small, deeply-impregnated granule (see Pl. 17, fig. 2), may be seen inside the nucleus, within the chromatin reticulum, often in close proximity to the nucleolus. Its nature I do not know ; possibly it represents the nucleolinus of a degenerated nucleolus or an extruded nucleolinus.

The fact that several nucleolini are often found within a single nucleolus suggests that these bodies can undergo division. Often two may be seen lying in the closest proximity, while sometimes the nucleolinus appears as a dumb-bell-shaped body. In the cat, however, these intra-nucleolar inclusions are so minute that observations on their division are very difficult.

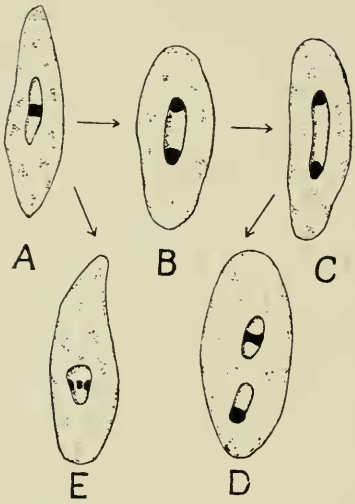
In addition to the Cajal technique, control observations were made after fixation in Champy's fluid and staining in Heidenhain's iron-hæmatoxylin. It was found that after prolonged differentiation bodies within the nucleoli, exactly similar in size, etc., to those impregnated by the silver method, could be demonstrated. Owing to their affinity for the hæmatein being almost equal to that of the surrounding nucleolar material their differentiation is no easy matter, and is best controlled with a water-immersion lens.

(B) The Nucleolinus in the Frog (Rana temporaria).—The body was demonstrated by the modified Cajal technique described above. The nucleolinus is larger than in the cat, being approximately 1 to 1·5 μ in diameter. Its shape also is more variable. Rarely are there more than two nucleolini per nucleolus, while the latter usually number one or two in each nucleus.

The nucleoli of the columnar cells of the frog's intestine are plasmosomes, as is evidenced by their oxyphility after staining with hæmatoxylin and eosin, Mann's methyl blue-eosin, and pyronin-methyl green. In the nucleoli depicted in Pl. 17, figs. 8 and 9, the nucleoli were bright red, the nucleolini black, and the chromatin green. In shape the nucleoli are generally round or oval (Pl. 17, figs. 6 to 9).

There seems but little doubt that the nucleoli are capable of multiplication. The most usual mode is apparently by transverse fission, the nucleolus elongating until it is a rodlet with rounded ends, and then dividing transversely. That the nucleolinus participates in the fission of the nucleolus is very probable; it may often be seen to have a median constriction;

TEXT-FIG.



A to D show the usual mode of multiplication of the nucleolus and the nucleolinus. The former elongates and finally divides transversely. The nucleolinus also divides, a portion of it being distributed to each of the daughter-nucleoli. Fig. E shows division of the nucleolinus unaccompanied by that of the nucleolus. Fragmentation of this sort is relatively rare. All the figures represent nuclei of columnar cells from the intestine of the frog. The nucleolini are black. Magnification circa 2690 diameters.

often there are two nucleolini lying in the closest proximity within the nucleolus; or they may be situated one towards each end of it. These stages may be easily observed in impregnated material counterstained with safranin. There does not appear to be any fixed stage in the process of nucleolar division at which fission of the nucleolinus takes place. The latter, in fact, frequently divides into two within

a nucleolus which is apparently unprepared for multiplication. But nevertheless there does seem to be a continuity of the substance of the nucleolinus during the division of the nucleolus.

The fact that in well-impregnated pieces of epithelium the nucleoli, when they number two per nucleus, always contain each a nucleolinus, is strongly suggestive of the continuity of these bodies in daughter-nucleoli.

Reference to the Text-figure will, I hope, make this clear.

Control observations, after fixation in Bouin's or Champy's fluid and staining in toluidin blue-eosin after the former, and iron-hæmatoxylin after the latter, demonstrate the nucleolinus (see Pl. 17, fig. 14).

In addition, the nucleoli were studied intra-vitam and with intra-vitam stains; of these pyronin of 1 in 1000 in frog Ringer stained the nucleolus very sharply. But the latter always appeared to be perfectly homogeneous with all methods.

Examination with the dark-ground illumination also failed to demonstrate the nucleolinus. The probable reason for the failure of intra-vitam observations to demonstrate the nucleolinus is that the refractive index of this body is the same as that of the nucleolus. There are, of course, other instances of intra-cellular structures rarely visible intra-vitam, e.g. the Golgi apparatus.

5. The Nucleolinus during Mitosis.

The study of the behaviour of the nucleolinus during cell-division was restricted to the intestinal epithelium of the frog. For here the nucleolinus is comparatively large, while its impregnation is less capricious and more selective than in the case of the cat. The last is an important point, for in the majority of the intestinal cells examined (including all the mitotic figures observed) the nucleolini were the only impregnated elements within the cells. There could thus be no

question of the nucleolini being confused with Golgi apparatus or mitochondrial elements.

In the early prophases of mitosis the nucleolus disappears in so far as can be ascertained by ordinary cytological technique. Nor does it become again apparent until the separation of the daughter-cells is complete.

Now the study of Cajal material, treated by the modified technique and counterstained with safranin, demonstrates the behaviour of the nucleolinus during cell-division. In the early prophase of mitosis, as drawn in Pl. 17, fig. 10, the spireme is beginning to undergo segmentation. There are two dumb-bell-like nucleolini, which, judging by their shape, are about to divide. Fig. 11 depicts a somewhat oblique section across an equatorial plate. The chromosomes are as yet undivided, but the bodies now form two pairs. The stage represented is a late prophase. Fig. 12 shows an early anaphase; the arrows indicate the direction of mitosis, for the section is somewhat oblique. The two chromosome groups, each with a pair of nucleolini, are nevertheless clearly visible. In fig. 15 a late anaphase is depicted, the two groups of daughter-chromosomes are far apart, the interzonal fibres being faintly visible between them. Each group contains two nucleolini, the upper right-hand one appearing to be about to divide. Fig. 16 shows a still later (telophase) stage. The nuclear membrane has re-formed, the chromatin is beginning to become differentiated into isolated condensations, while the nucleolini are clearly visible as a pair of granules in each daughter-nucleus. In fig. 13, which represents a transverse section of the equatorial plate of a prophase, it will be seen that there are only two nucleolini. I have observed this in other prophases, and have come to the conclusion that in all probability the division of the nucleolini often may not occur until the later stages of the prophase. The presence of two nucleolini in the prophase stage is strongly suggestive of the derivation of each from a separate nucleolus, for the majority of nuclei of resting cells contain two of these bodies.

It must be noted that in the original preparations the

nucleolini are impregnated black in a perfectly specific manner, while the chromosomes are red and the cytoplasm pinkish—colour contrasts impossible to indicate in pen-and-ink drawings. Nor has any attempt been made to count and depict the exact chromosome number, as, apart from the fact that this would be without any direct bearing on these observations, the safranin does not give a sufficiently precise stain to enable counts to be made on material which has been previously subjected to the Cajal technique.

A comparison of the size of the nucleolini in resting and dividing cells shows that the former become distinctly smaller during mitosis. It may be added that the nucleolinus was observed in both recently metamorphosed and fully grown frogs.

6. Discussion.

It is, of course, impossible to make sweeping generalisations on the nature of the nucleolinus from observations on it in only two different species of animals.

Nevertheless, the fact that such a body can be demonstrated by the Cajal method indicates that it must be of a different chemical nature to the surrounding nucleolar substance, since it possesses, under certain conditions, a specific affinity for silver not shared by the other nuclear constituents.

Furthermore, that the nucleolinus should be present in both karyosomes (in the cat's intestine) and in plasmosomes (in that of the frog) shows that this body is not peculiar to one of these types of nucleoli.

From the behaviour of the nucleolinus during mitosis I believe that there is undoubted continuity of its substance from one cell generation to another in the case of the columnar cells of the frog's intestine. This, of course, is in marked contrast with the fate of the plasmosome with which it is associated, the latter body apparently disappearing during cell division. Montgomery (**11**), with regard to this, says: "There is no substantial basis for Zimmermann's conclusion 'omnis nucleolus e nucleolo,' or more strictly speaking,

that the nucleolus in most cases is not derived from a previously existing one." By the term "nucleolus," be it noted, Montgomery means a plasmosome.

As to the significance of the nucleolinus, it is possible that this body initiates the formation of the plasmosome around itself at the completion of mitosis. If this be so, the nucleolinus would be, so to speak, the precursor of the nucleolus. In this case Zimmermann's statement (**14**), "omnis nucleolus e nucleolo," would be true, in so far as continuity of a specialised portion of it—the nucleolinus—was concerned.

The only intra-nucleolar bodies which can, with any degree of certainty, be homologised with the structures described in this paper, are the minute granules or rodlets discovered by P. Del Rio Hortega (**9**) in the nucleoli of ovarian oöcytes (see p. 381). This observer used the same technique as myself.

7. Summary.

(1) The presence of one or more intra-nucleolar bodies in the columnar cells of the intestinal epithelium in the frog and cat has been demonstrated. To these inclusions the term "nucleolinus," originally introduced by Haeckel, has been applied.

(2) By a modification of the Cajal technique for the Golgi apparatus, the nucleolinus has, in the frog's intestine, been impregnated in a perfectly specific manner.

(3) The presence of nucleolini both in karyosomes (e.g. cat) and in plasmosomes (e.g. frog) in the columnar intestinal cells shows that they are not restricted to one of these types of nucleolus.

(4) Evidence has been adduced showing that division of the nucleolus (plasmosomes) in the resting cell is in all probability accompanied by fission of the nucleolinus.

(5) During mitosis the nucleolinus has been shown apparently to divide, a portion or portions of it being distributed to each of the daughter-nuclei. This is in contrast to the behaviour of the nucleolus (plasmosome), which disintegrates during cell division.

(6) The presence of the nucleolinus has been confirmed in the columnar cells of the intestinal mucosa in both the frog and the cat by other and simpler methods than the Cajal technique.

(7) The significance of the apparent continuity of the nucleolinus has been very briefly discussed.

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